

specific for the target sequence, and that the presence of genomic DNA does not cause any significant background cleavage.

In Figure 35, lanes 2 and 3 contain reaction products from reactions containing the single-stranded target DNA (M13mp18), the probe (SEQ ID NO:43) and the invader oligonucleotide (SEQ ID NO:46) in the absence or presence of human genomic DNA, respectively. Examination of lanes 2 and 3 demonstrate that the invader detection assay may be used to detect the presence of a specific sequence on a single-stranded target molecule in the presence or absence of a large excess of competitor DNA (human genomic DNA).

In Figure 35, lanes 4 and 5 contain reaction products from reactions containing the double-stranded target DNA (M13mp19), the probe (SEQ ID NO:43) and the invader oligonucleotide (SEQ ID NO:46) in the absence or presence of human genomic DNA, respectively. Examination of lanes 4 and 5 show that double stranded target molecules are eminently suitable for invader-directed detection reactions. The success of this reaction using a short duplexed molecule, M13mp19, as the target in a background of a large excess of genomic DNA is especially noteworthy as it would be anticipated that the shorter and less complex M13 DNA strands would be expected to find their complementary strand more easily than would the strands of the more complex human genomic DNA. If the M13 DNA reannealed before the probe and/or invader oligonucleotides could bind to the target sequences along the M13 DNA, the cleavage reaction would be prevented. In addition, because the denatured genomic DNA would potentially contain regions complementary to the probe and/or invader oligonucleotides it was possible that the presence of the genomic DNA would inhibit the reaction by binding these oligonucleotides thereby preventing their hybridization to the M13 target. The above results demonstrate that these theoretical concerns are not a problem under the reaction conditions employed above.

In addition to demonstrating that the invader detection assay may be used to detect sequences present in a double-stranded target, these data also show that the presence of a large amount of non-target DNA (470 ng/20 µl reaction) does not lessen the specificity of the cleavage. While this amount of DNA does show some impact on

the rate of product accumulation, probably by binding a portion of the enzyme, the nature of the target sequence, whether single- or double-stranded nucleic acid, does not limit the application of this assay.

EXAMPLE 15

5 Signal Accumulation In The Invader-Directed
Cleavage Assay As A Function Of Target Concentration

To investigate whether the invader-directed cleavage assay could be used to indicate the amount of target nucleic acid in a sample, the following experiment was performed. Cleavage reactions were assembled which contained an invader oligonucleotide (SEQ ID NO:46), a labelled probe (SEQ ID NO:43) and a target nucleic acid, M13mp19. A series of reactions, which contained smaller and smaller amounts of the M13 target DNA, was employed in order to examine whether the cleavage products would accumulate in a manner that reflected the amount of target DNA present in the reaction.

The reactions were conducted as follows. A master mix containing enzyme and buffer was assembled. Each 5 µl of the master mixture contained 25 ng of Cleavase® BN nuclease in 20 mM MOPS (pH 7.5) with 0.1% each of Tween 20 and NP-40, 4 mM MnCl₂ and 100 mM KCl. For each of the cleavage reactions shown in lanes 4-13 of Figure 36, a DNA mixture was generated which contained 5 pmoles of the fluorescein-labelled probe oligonucleotide (SEQ ID NO:43), 50 pmoles of the invader oligonucleotide (SEQ ID NO:46) and 100, 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01 or 0.005 fmole of single-stranded M13mp19, respectively, for every 5 µl of the DNA mixture. The DNA solutions were covered with a drop of ChillOut® evaporation barrier (MJ Research) and brought to 61°C. The cleavage reactions were started by the addition of 5 µl of the enzyme mixture to each of tubes (final reaction volume was 10 µl). After 30 minutes at 61°C, the reactions were terminated by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes. Samples were heated to 90°C for 1 minutes immediately before electrophoresis through a 20% denaturing acrylamide

gel (19:1 cross-linked) with 7 M urea, in a buffer containing 45 mM Tris-Borate (pH 8.3), 1.4 mM EDTA. To provide reference (*i.e.*, standards), 1.0, 0.1 and 0.01 pmole aliquots of fluorescein-labelled probe oligonucleotide (SEQ ID NO:43) were diluted with the above formamide solution to a final volume of 18 μ l. These reference markers were loaded into lanes 1-3, respectively of the gel. The products of the cleavage reactions (as well as the reference standards) were visualized following electrophoresis by the use of a Hitachi FMBIO fluorescence imager. The results are displayed in Figure 36.

In Figure 36, boxes appear around fluorescein-containing nucleic acid (*i.e.*, the cleaved and uncleaved probe molecules) and the amount of fluorescein contained within each box is indicated under the box. The background fluorescence of the gel (see box labelled "background") was subtracted by the fluoro-imager to generate each value displayed under a box containing cleaved or uncleaved probe products (the boxes are numbered 1-14 at top left with a V followed by a number below the box). The lane marked "M" contains fluoresceinated oligonucleotides which served as markers.

The results shown in Figure 36, demonstrate that the accumulation of cleaved probe molecules in a fixed-length incubation period reflects the amount of target DNA present in the reaction. The results also demonstrate that the cleaved probe products accumulate in excess of the copy number of the target. This is clearly demonstrated by comparing the results shown in lane 3, in which 10 fmole (0.01 pmole) of uncut probe are displayed with the results shown in 5, where the products which accumulated in response to the presence of 10 fmole of target DNA are displayed. These results show that the reaction can cleave hundreds of probe oligonucleotide molecules for each target molecule present, dramatically amplifying the target-specific signal generated in the invader-directed cleavage reaction.